Integrin-dependence of Lymphocyte Entry into the Splenic White Pulp

Charles G. Lo,1 Theresa T. Lu,1,2 and Jason G. Cyster1

1Howard Hughes Medical Institute and Department of Microbiology and Immunology and 2Division of Pediatric Immunology and Rheumatology, University of California San Francisco, San Francisco, CA 94143

Abstract

The steps involved in lymphocyte homing to the white pulp cords of the spleen are poorly understood. We demonstrate here that the integrins lymphocyte function associated (LFA)-1 and α4β1 make essential and mostly overlapping contributions necessary for B cell migration into white pulp cords. T cell entry to the white pulp is also reduced by blockade of LFA-1 and α4β1. The LFA-1 ligand, intercellular adhesion molecule 1 is critical for lymphocyte entry and both hematopoietic cells and radiation-resistant cells contribute to this requirement. Vascular cell adhesion molecule 1 contributes to the α4β1 ligand requirement and a second ligand, possibly fibronectin, also plays a role. By contrast with the entry requirements, antigen-induced movement of B cells from follicles to the outer T zone is not prevented by integrin blocking antibodies. Comparison of the distribution of integrin-blocked B cells and B cells treated with the Gαi inhibitor, pertussis toxin, early after transfer reveals in both cases reduced accumulation in the inner marginal zone. These observations suggest that chemokine receptor signaling and the integrins LFA-1 and α4β1 function together to promote lymphocyte transit from the marginal zone into white pulp cords.

Key words: splenic white pulp • LFA-1 • α4β1 • lymphocyte homing • marginal zone

Introduction

The spleen is a major secondary lymphoid tissue important in host protection against many types of pathogen, especially encapsulated bacteria (1). The lymphoid regions of the spleen, known as white pulp cords, are organized into inner T cell zones and outer B cell follicles. The follicular areas associated with large white pulp cords are surrounded by a region termed the marginal zone, which consists of loosely associated B cells, macrophages, and reticular cells and forms the border between the white pulp and the red pulp (2). Many small follicular arterioles terminate in the marginal zone in a region known as the marginal sinus whereas a smaller number of penicillar arterioles terminate directly in the red pulp (3, 4). Although there is some controversy regarding whether a marginal sinus exists in the human spleen, corrosion casting experiments established that large amounts of blood are released into sinuses present within the marginal zone in all species examined, including humans and rodents (4, 5). Many of the lymphocytes entering the spleen are released in the marginal zone (6–8).

Some of these cells, together with the nonlymphocytes, pass to the outer region of the marginal zone and then to the red pulp or directly into venous sinuses. A fraction of the lymphocytes take a different route and quickly begin appearing within the B and T cell areas of the white pulp cords (7, 8).

Extensive studies of the molecular mechanisms controlling lymphocyte entry into lymph nodes and Peyer’s patches have established a cascade model of entry. First, lymphocytes undergo rolling interactions, principally mediated by L-selectin binding to ligands on the high endothelial venules (HEVs;* references 9 and 10). Subsequently, a chemokine-mediated triggering event occurs that causes integrin activation and adhesion. LFA-1 (αLβ2 or CD11a/CD18), α4β7, and α4β1 contribute to differing extents to this integrin adhesion requirement (10, 11).

Despite the detailed understanding of the steps involved in lymphocyte entry to lymph nodes and Peyer’s patches,

*Abbreviations used in this paper: AP, alkaline phosphatase; CFSE, 5(and 6)-carboxyfluorescein diacetate succinimidyl ester; HEL, hen egg lysozyme; HEV, high endothelial venule; HRP, horseradish peroxidase; ICAM, intercellular adhesion molecule; MAdCAM, mucosal addressin cell adhesion molecule; PTX, pertussis toxin; VCAM, vascular cell adhesion molecule.
relatively little is known about how the cells enter white pulp cords in the spleen (12, 13). In particular, whether integrins are required has not been established, although some experiments have suggested that they may not be critical (14–16). However, our recent finding that marginal zone B cell adhesion within the splenic marginal zone involves redundant contributions by the integrins LFA-1 and α4β1 (17) led us to test the combined contribution of these integrins in lymphocyte entry to white pulp cords.

Materials and Methods

Mice and Adoptive Cell Transfer. C57BL/6 (B6) mice were from Charles River Laboratories or a colony maintained at University of California San Francisco. Igα (Ig heavy chain of α) Thy1.1-GPIIb-B6 mice (termed Igh7B6 mice) were from The Jackson Laboratory. β2−/− (18), β7−/− (19), and intercellular adhesion molecule (ICAM)-1−/− mice (20) were obtained on a B6 background from The Jackson Laboratory. Igκ transgenic B6 mice express IgMκ and IgDκ specific for hen egg lysozyme (HEL). Mice were treated with antibodies by intraperitoneal injection in 300 μL PBS. Bone marrow chimeras were made as previously described (17). For adoptive transfers, mice were injected with ~3 × 10^7 spleen cells in 0.3 mL medium. In some experiments, cells were labeled before transfer with 5(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) as previously described (21).

Antibodies and Treatments. The anti-α (clone M17/4, rat IgG2a) hybridoma was from American Type Culture Collection and the anti-α4 (clone PS/2, rat IgG2b) hybridoma was provided by David Erle (University of California San Francisco, San Francisco, CA). Anti–vascular cell adhesion molecule (VCAM)-1 (clone M/K-2, rat IgG1) was obtained from Southern Biotechnology Associates, Inc. Anti–mucosal addressin cell adhesion molecule (MAdCAM)-1 (clone M/K-2, rat IgG1) was obtained from Southern Biotechnology Associates, Inc. Anti–mucosal addressin cell adhesion molecule (MAdCAM)-1 (clone MECA-367, rat IgG2A) and anti–β1 (clone HA2/5, Armenian hamster IgM) were obtained from staining antibodies and the subsequent trafficking of transferred lymphocytes was examined. Strikingly, in mice given the combined treatment, B cell entry to white pulp cords was reduced by ~90% (Fig. 1, A and C) and T cell entry was reduced by 50% (Fig. 1 B). As expected (17), the combined antibody treatment also led to displacement of marginal zone B cells from around the white pulp cords (Fig. 1, A and B) whereas marginal metallophilic macrophages and marginal zone macrophages were not displaced (unpublished data). The total number of transferred lymphocytes in the spleen was not greatly affected by the antibody treatment whereas entry into lymph nodes was strongly inhibited (Fig. 1 C), as expected (10, 11). The lack of an effect on total spleen cell numbers despite the inhibition in cell entry to white pulp cords is similar to the previous findings for cells treated with PTX (12) and most likely reflects the presence of greater numbers of cells in the red pulp due to the lymphocytosis caused by the block in entry to lymph nodes. Analysis of the effect of blocking LFA-1 alone revealed ~50% inhibition in white pulp accumulation of B cells (Fig. 1 C) and ~30% inhibition for T cells (not depicted). In a recent study, cells from LFA-1−/− deficient mice were reported to show a small (~20%) reduction in lymphocyte homing to white pulp cords (16). The differences

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Results and Discussion

LFA-1 and α4β1 Function in B and T Cell Entry to the Splenic White Pulp. To test the possibility that LFA-1 and α4-containing integrins make overlapping contributions to lymphocyte entry into the splenic white pulp, WT mice were treated with a combination of LFA-1– and α4 blocking antibodies and the subsequent trafficking of transferred lymphocytes was examined. Strikingly, in mice given the combined treatment, B cell entry to white pulp cords was reduced by ~90% (Fig. 1, A and C) and T cell entry was reduced by 50% (Fig. 1 B). As expected (17), the combined antibody treatment also led to displacement of marginal zone B cells from around the white pulp cords (Fig. 1, A and B) whereas marginal metallophilic macrophages and marginal zone macrophages were not displaced (unpublished data). The total number of transferred lymphocytes in the spleen was not greatly affected by the antibody treatment whereas entry into lymph nodes was strongly inhibited (Fig. 1 C), as expected (10, 11). The lack of an effect on total spleen cell numbers despite the inhibition in cell entry to white pulp cords is similar to the previous findings for cells treated with PTX (12) and most likely reflects the presence of greater numbers of cells in the red pulp due to the lymphocytosis caused by the block in entry to lymph nodes. Analysis of the effect of blocking LFA-1 alone revealed ~50% inhibition in white pulp accumulation of B cells (Fig. 1 C) and ~30% inhibition for T cells (not depicted). In a recent study, cells from LFA-1−/− deficient mice were reported to show a small (~20%) reduction in lymphocyte homing to white pulp cords (16). The differences
in the extent of inhibition observed might reflect our direct enumeration of cells within white pulp cords versus Nolte et al.’s (22) approach of measuring cells flow cytometrically in enzymatically isolated spleen preparations enriched for white pulp cord cells. Treatment with \( \alpha_4 /H9251 \) blocking antibodies alone had no measurable effect on the number of B or T lymphocytes accumulating in the white pulp over the 3-h period (Fig. 1). Thus, the contribution of \( \alpha_4 \)-containing integrins to lymphocyte homing to the white pulp appears to be fully redundant to LFA-1 whereas the contribution of LFA-1 is partially redundant with the \( \alpha_4 \)-containing integrins.

As B cell homing was more strongly inhibited by integrin neutralization than T cell homing, we focused our

Figure 1. LFA-1 and \( \alpha_4 \beta_1 \) function in B and T cell entry into splenic white pulp cords. (A and B) Immunohistochemical analysis of spleen sections from B6 mice that had received WT Ig"Thy1" spleen cells 3 h before and had been pretreated with PBS or \( \alpha_L \) and \( \alpha_4 \) neutralizing antibodies, as indicated, 1 h before cell transfer. Transferred B cells were detected by IgM+ plus IgD+ staining (A, blue), transferred T cells by Thy1+ staining (B, blue), and endogenous B cells by B220 staining (brown). ×5. (C) Summary of B cell homing data showing the average number of transferred B cells per white pulp cross section (left), per one fifth of spleen (middle), and in a pool of inguinal and brachial lymph nodes (right). Donor cells were from WT, \( \beta_2^{-/-} \), or \( \beta_7^{-/-} \) mice as indicated. Each bar shows the average (±SD) value for data from at least four animals of each type except for the \( \beta_2^{-/-} \) and \( \beta_7^{-/-} \) transfers where the individual data points are denoted by *; \( * \), \( P < 0.05 \) compared with untreated WT controls. Similar enumeration was performed for T cells and the following average number of cells were detected per white pulp cord: PBS-treated, 218 ± 11 (n = 3); \( \alpha_4 \)-treated, 242 (n = 1); \( \alpha_L \)-treated, 152 ± 13 (n = 3); \( \alpha_L \) plus \( \alpha_4 \)-treated, 118 ± 14 (n = 4). (D and E) Immunohistochemical analysis of spleen sections from WT Ig"B6 mice that had received \( \beta_7^{-/-} \) Ig"B6 cells (D) or B6 mice that had received WT Ig"B6 spleen cells (E) and had been treated with integrin neutralizing antibodies, as indicated. Transferred B cells were detected by staining IgM+ plus IgD+ (D) or IgM+ plus IgD+ (E).
of VCAM-1 function was associated with a reduction in B cell homing to the white pulp compared with animals treated with LFA-1 blocking antibodies alone (Fig. 2, A and B). However, the block in homing by the combination of anti-VCAM-1 and anti-LFA-1 was less severe than that observed after anti-α4 plus anti-LFA-1 treatment or anti-β1 plus anti-LFA-1 treatment (Fig. 1), indicating that an additional α4β1 ligand participates in B cell homing into white pulp cords. Combined treatment with neutralizing antibodies specific for VCAM-1, MadCAM-1, and LFA-1 did not cause any greater inhibition than observed by VCAM-1 and LFA-1 blocking (Fig. 2 B). Although no reagents are available to selectively inhibit α4β1–fibronectin interactions in the mouse, these findings are consistent with the possibility that fibronectin functions as an adhesion molecule for B cell migration into white pulp cords. However, our experiments do not rule out possible contributions by other molecules that can bind α4β1, such as von Willebrand factor and thrombospondin (24). Favoring a role for fibronectin, immunohistochemical analysis revealed that this extracellular matrix protein is expressed at a high level throughout the marginal zone as well as in the red pulp (Fig. 2 C). Analysis of sections double stained for fibronectin and MadCAM-1 indicated an overlap in the staining pattern consistent with fibronectin being associated with marginal sinus lining cells (Fig. 2 C). Fibronectin has also been identified within the marginal zone of human spleen (25).

To determine whether the ICAM-1 necessary for B cell homing in the spleen was made by hematopoietic or non-hematopoietic cells, radiation bone marrow chimeras were generated. After reconstitution, these animals were treated with α4 blocking antibodies and then transferred with WT lymphocytes (Fig. 2). For reasons that are unclear, the homing of B cells to white pulp cords in control bone marrow recipients was only weakly reduced compared with the WT controls (Fig. 2 D), indicating that there was some alteration in B cell trafficking due to the effects of irradiation. However, consistent with this control group, homing in ICAM-1–deficient chimeras (ICAM-1<sup>−/−</sup> bone marrow → ICAM-1<sup>−/−</sup> recipients) was severely inhibited (Fig. 2 D). By contrast, in mice where ICAM-1 expression was restricted to radiation-resistant cells (ICAM-1<sup>−/−</sup> bone marrow → WT recipients), B cell entry to white pulp cords was only weakly reduced compared with the WT controls. Similarly, when ICAM-1 expression was restricted to radiation-sensitive hematopoietic cells (WT bone marrow → ICAM-1<sup>−/−</sup> recipients), homing was only weakly affected (Fig. 2 D). Therefore, the ICAM-1 requirement for B cell homing into splenic white pulp cords involves contributions by both radiation-sensitive hematopoietic cells and radiation-resistant cells. Consistent with a hematopoietic cell type playing a role in guiding lymphocyte homing in the spleen, ablation of marginal zone macrophages was associated with a reduction in the efficiency of lymphocyte homing into white pulp cords (26). The nature of the ICAM-1–expressing radiation-resistant cells in the marginal zone is unclear.
Figure 2. ICAM-1 and VCAM-1 function in B cell entry into splenic white pulp cords. (A) Immunohistochemical analysis of spleen sections from ICAM-1−/− or WT mice that had received Igha B6 spleen cells 3 h before and had been pretreated as indicated 1 h before cell transfer. Transferred cells were detected as described in Fig. 1. (B) Summary of transferred B cell homing data showing the average number of B cells per white pulp cross section (left), per one fifth of spleen (middle), and the frequency in a pool of inguinal and brachial lymph nodes (pLN) or in mesenteric lymph nodes (mLN) as indicated (right). Donor cells were from WT mice and the genotype of the recipient animals is indicated. The dashed line in the White Pulp panel indicates the average number of transferred WT cells reaching the white pulp in untreated controls as shown in Fig. 1. * P < 0.05 compared with untreated WT controls; ** P < 0.05 compared with ICAM-1−/− or anti-α4−treated mice. (C) Fibronectin expression pattern in mouse spleen. Spleen sections from WT mice were stained to detect fibronectin alone (left, brown) or together with MAdCAM-1 (right, blue). (D) Summary of B cell homing data in ICAM-1−/− or control bone marrow chimeras treated with α4 blocking antibody. Recipient ICAM-1−/− or WT mice were reconstituted with ICAM-1−/− or WT bone marrow (BM), as indicated, treated with anti-α4, and transferred with WT cells. * P < 0.05 compared with WT bone marrow chimeras. Cell number was enumerated as described in Materials and Methods. Each bar shows the average (±SD) value for data from at least four animals per group in B and three animals in D.
but may include the marginal sinus lining cells and marginal zone reticular cells (2, 5, 17).

Relocalization of Antigen-engaged B Cells from Follicles to T Zones Is Not Blocked by Integrin Neutralizing Antibodies. To examine whether integrins are also critical for B cell movement within the white pulp, we tested the effect of LFA-1 and α4 blocking antibodies on the migration of antigen-engaged B cells from follicles to the outer T zone (21). Previous experiments have established that this redistribution process is complete within 6 h of antigen exposure. Therefore, IgHEL transgenic B cells specific for HEL were transferred to WT mice and after allowing 1 d for the cells to become distributed in lymphoid follicles, mice were treated with LFA-1 and α4 neutralizing antibodies and then exposed to HEL antigen. Immunohistochemical analysis of spleens isolated 6 h later demonstrated that B cells had redistributed from the follicles to T cell areas in a manner similar to mice that did not receive integrin blocking antibodies (Fig. 3 A). Samples of the tissue from each animal were used to generate cell suspensions and these were tested by flow cytometric analysis to confirm that the integrins on both the endogenous and transferred B cells were saturated by the neutralizing antibodies (Fig. 3 B). These findings indicate that movement of B cells inside the splenic white pulp can occur when LFA-1 and α4β1 are blocked, favoring the conclusion that the integrin requirement for homing into the white pulp reflects a role during an early entry step. Additional approaches will be needed to determine whether integrins contribute to the basal motility of naive lymphocytes within the white pulp and to identify what other types of adhesive interactions are used by migrating white pulp cells.

PTX and Integrin Blocking Antibodies Inhibit Splenic B Cell Migration at a Similar Step. Previous experiments have demonstrated that PTX-treated lymphocytes fail to enter white pulp cords (12, 13). To examine whether the requirements for Goi signaling and integrin function occur at a similar step, short-term transfer experiments were performed in which mice received cotransfers of treated and control cells (Fig. 4 A). At early time points after transfer of untreated cells, many cells were present within the marginal zone and, similar to a previous report (7), a fraction of the cells appeared on the inner (follicular) edge of the marginal zone associated with the MAdCAM-1+ cells (Fig. 4 B). Comparison of the distribution of PTX-treated cells and control cells revealed that fewer of the treated cells were located along this boundary whereas treatment with the inactive oligomer B subunit of PTX had no effect (Fig. 4, B, C and E). A similar analysis was attempted to test the role of integrins at this step by transferring cells that had been pretreated with integrin blocking antibodies together with untreated cells, but no differences were observed in the distribution of treated and untreated cells (unpublished data). However, flow cytometric analysis of the pretreated cells isolated 10 min after transfer revealed that ~50% of the surface integrins were no longer occupied by neutralizing antibody despite pretreatment before transfer (not depicted). Therefore, we used an alternative approach where β2-deficient and WT cells were cotransferred to mice that had been pretreated with integrin blocking antibodies together with untreated cells. Although all the cells would have their α4 integrins neutralized, the experiments described above established that this does not measurably affect passage of WT lymphocytes into white pulp cords (Fig. 1). Analysis of the distribution of β2−/− versus control cells 15 min after transfer revealed a defi-

![Figure 3](image-url)
ciency in B2^−/− cells near the inner edge of the marginal zone (Fig. 4, D and E). Therefore, treatment with PTX and combined inhibition of α4β1 and LFA-1 both disrupt attachment or accumulation of B cells on the inner side of the marginal zone, as well as prevent their subsequent appearance within the white pulp cords.

**Concluding Remarks.** The findings described above demonstrate that LFA-1 and α4β1 and their ligands play...
critical roles in lymphocyte entry into the splenic white pulp, revealing closer parallels than previously thought between this process and entry into lymph nodes and Peyer's patches. The integrin entry requirement is stronger for B than T cells, and LFA-1 appears to make the dominant contribution to the homing requirement for both cell types. Overlapping contributions of LFA-1 and α4β1 have previously been observed for lymphocyte attachment to HEVs and accumulation in the bone marrow (11) and for marginal zone B cell lodgement in the marginal zone (17).

Our findings indicate that the key ligands involved in B cell entry are ICAM-1, VCAM-1, and a second α4β1 ligand. As fibronectin is present in large amounts within the marginal zone, we suggest that this α4β1 ligand also contributes to the entry process. Although MadCAM-1 is expressed in an appropriate location to participate in entry (15), experiments using MadCAM-1 blocking antibodies and β7-deficient cells failed to reveal a contribution for this adhesion molecule even when the contributions of LFA-1/ICAM-1 were blocked. Consistent with MadCAM-1 not having a critical role, this ligand has not been detected in rat spleen (27). The requirement of α4β1 and LFA-1 both for cell entry into the white pulp and for long-term adherence of cells in the marginal zone might be explained at least in part by marginal zone B cells expressing higher levels of functional integrins and adhering more strongly to ICAM-1 and VCAM-1 than follicular cells (17). It may also indicate that other differences between marginal zone and follicular B cells contribute to positioning, such as differences in chemokine responsiveness.

Although our experiments indicate that Gαi signaling and integrins participate at a similar early step in white pulp entry, we have not yet been able to determine the order of their requirement. Our findings are consistent with the possibility that integrins function both upstream and downstream of chemokine signals during the entry process. Selectins do not appear to be required for lymphocyte entry to the splenic white pulp (16) perhaps because the shear forces operating on lymphocytes released from splenic arterioles in the red pulp (8, 30). It seems possible that the integrin requirements for entry by this pathway will be distinct and that T and B cells may differ in the degree to which they use these routes to enter the white pulp cords.

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